Preparation of gold nanoparticles-α lactalbumin binary complex for Breast Cancer therapy

A PROJECT THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE IN

BACHELOR OF TECHNOLOGY IN BIOTECHNOLOGY ENGINEERING

By

Anjul Khadria
(Roll No. - 108BT013)

DEPARTMENT OF BIOTECHNLOGY AND MEDICAL ENGINEERING

NATIONAL INSTITUTE OF TECHNOLOGY ROURKELA

ROURKELA-769008, ODISHA, INDIA

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Under the Guidance of
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NATIONAL INSTITUTE OF TECHNOLOGY ROURKELA

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National Institute Of Technology, Rourkela

CERTIFICATE

This is to certify that project entitled "PREPARATION OF GOLD NANOPARTICLES-α LACTALBUMIN BINARY COMPLEX FOR BREAST CANCER THERAPY" submitted by ANJUL KHADRIA (Roll No. – 108BT013), in partial fulfillment of the requirements for the award of Bachelor of Technology in Biotechnology Engineering at National Institute of Technology, Rourkela (Deemed University) is an authentic work carried out by her under my supervision and guidance.

To the best of my knowledge, the matter embodied in the Project report has not been submitted to any other University/Institute for the award of any Degree or Diploma.

Date: (Dr. Subhankar Paul)

Place: Rourkela Department of Biotechnology & Medical Engineering

National Institute of Technology, Rourkela

Rourkela-769008, ODISHA

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Anjul Khadria

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Contents

List of Figures

List of Tables

Abstract

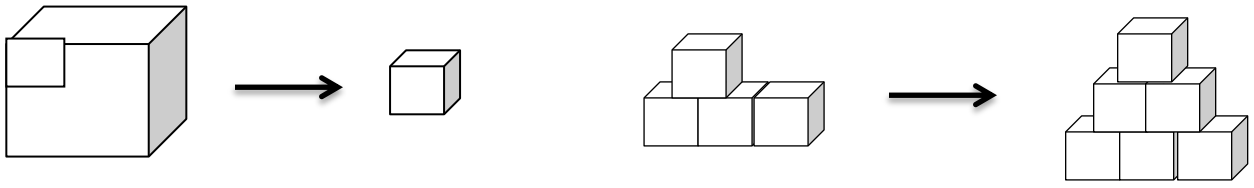
Gold nanoparticles are used normally for biomedical application like cancer diagnosis and have been used as drug carriers. α-lactalbumin (α-lac) protein has been observed to have anti-cancerous potential. Monodispersed and stable gold nanoparticles were synthesized by three different methods: microemulsion process, green synthesis from *Cannabis indica* leaf (bhang) extract and citrate reduction process. The nanoparticles were characterized by SEM (Scanning Electron Microscope), UV-Visible spectroscopy and DLS (Dynamic Light Scattering). The gold nanoparticles average sizes were found to be 10nm, 47nm and 140.6 nm synthesized from microemulsion, citrate reduction method and green synthesis respectively. It has been found that *Cannabis indica* leaf extract has the capability to act as a reducing agent during synthesis of gold nanoparticles. In the present investigation we have synthesized gold nanoparticle- α-lac protein conjugate which were used for breast cancer therapy. Gold nanoparticle- α-lac protein conjugates were prepared in vitro and their effect on breast cancer cell lines have been observed. Conjugates of gold nanoparticles prepared by bhang reduction method and α-lactalbumin protein showed better efficiency in the inhibition of proliferation of MDAMB-231 breast cancer cells than conjugates of gold nanoparticles prepared by citrate reduction method and α-lactalbumin protein. Nanophotothermolysis was also observed in the gold nanoparticles prepared from bhang reduction method in the presence of light.

Chapter 1

Introduction

1.1 Introduction

Materials when taken down to the size of nanometer range that is less than 100nm show a great change in their physical, chemical and optical properties from that of bulk material. The nanoparticles have found to be of profound use in different fields of biological sciences, electronics, automobile industry, chemical industry, in cosmetics, food industry etc. Different types of nanomaterials such as nanoparticles, nano-fibers, carbon nanotubes, nano films etc. have been synthesized and applied in the above mentioned fields. Due to their extensive use the synthesis and characterization of nanomaterials has become a needful task. Two major approaches for the synthesis of nanomaterials are: (i) Top- Down method (Figure 1) in which bulk materials are broken down to nanometer range and (ii) Bottom-Up method (Figure 1) in which different ions are aggregated to form materials of nanometer range. Different methods falling under these approaches are Sono-chemical method, Chemical Vapor Deposition, Electrophoretic Deposition, Electrospinning, Micro-emulsion method, Laser Ablation, Flame pyrolysis, heating stirring method, mechanical milling etc. These methods have different approaches depending upon the type of environment such as vapor phase, liquid phase or solid phase.



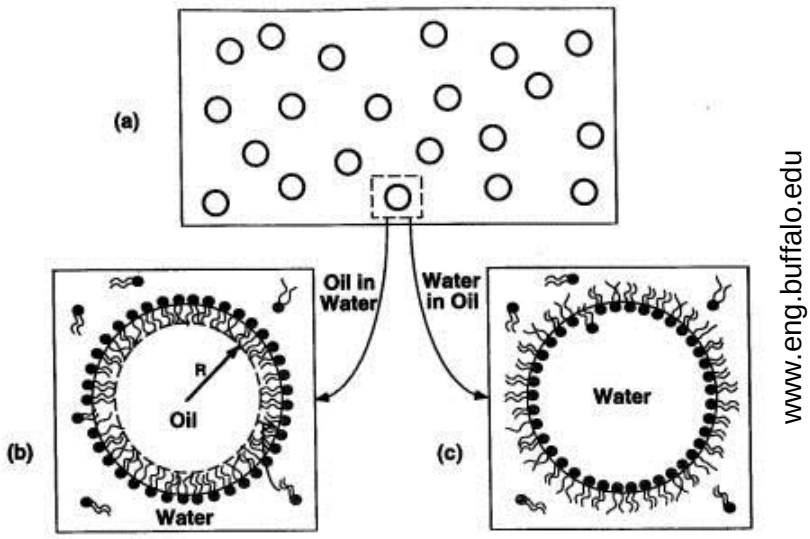
(i) Top- Down method (ii) Bottom-Up method

Figure 1 Approaches for synthesis of nanomaterials

Nanoparticles such as ZnO, Au, Ag, Pt etc. have been found to be of different applications in the field of biological sciences. Nanoparticles are used as drug carriers for cancerous cells, in gene therapy and as fluorescent agents for detecting cancerous cells [1]. Due to the non-toxic nature and inert core gold nanoparticles have found their way in the field of biological sciences. Gold nanoparticles are red in color due to the surface plasmon resonance because it scatter light in visible and near IR region. Due to its extensive physical, chemical and optical properties gold nanoparticles have seen about 4000 papers being published in its field in the last decade [2]. For the better application of these nanoparticles in most of the above mentioned fields it is desirable that the nanoparticles are monosized so that the interaction of the nanoparticles with drugs, proteins or cells does not alter. If the nanoparticles are polydispersed then the interaction cannot be defined properly as different nanoparticles with different sizes alter in their properties. For example nanoparticles of size 70nm will not show same result if interacted with protein as nanoparticles of 30nm. So in order to study the interaction pattern and its better application the size distribution of the nanoparticles synthesized should not be too high.

1.2 Microemulsion

For the synthesis of nanoparticles different methods have been developed. Use of microemulsion for synthesis of nanoparticles is a good method to synthesize monodispersed nanoparticles. Microemulsions are clear, thermodynamically stable mixture of two liquids in which one of the liquid is less in amount than the other in presence of surfactant and co-surfactant. Microemulsions (Figure 2)[3] are basically of two types that is water in oil (w/o) and oil in water (o/w). In water in oil type microemulsion oil is the dispersed phase while in oil in water type microemulsion water is the dispersed phase which are also known as reverse micelles and micelles respectively.



(a)Normal microemulsion (b) o/w microemulsion (c) w/o microemulsion

Figure 2 Types of microemulsion

The droplet size in the microemulsion is less than 100nm while when the droplet size is about 100nm-1000nm it is termed as miniemulsion. Microemulsions have droplets of equal size within which the nanoparticles are synthesized so the synthesis of monodispersed nanoparticles can be achieved easily by the use of microemulsion method.

1.3 α-lactalbumin protein

α-lactalbumin protein (α-lac) is a whey protein found in milk. Is molecular mass is 14kDa. The complex of α-lac with oleic acid has been found to be effective against cancer cells. The complex is known as BAMLET (Bovine α-lactalbumin Made LEthal to Tumors) and HAMLET (Human α-lactalbumin Made LEthal to Tumors) [4].

1.4 Gold nanoparticles

Gold nanoparticles are particles whose size varies from 10nm-100nm. All the three dimension of the particles are in nano dimension. Gold is a metal whose atomic weight is 79 and is inert in nature and so is gold nanoparticle. In 1857 Faraday showed the formation of gold nanoparticles first in a scientific way. Gold nanoparticles have been found to be of immense use in the field of biological sciences owing to its physical, chemical and optical properties. Due to its non-toxic nature it is used as catalyst, cell markers, drug delivery etc. Gold nanoparticles are also used in the diagnosis of cancer cells.

1.5 Cannabis indica

Cannabis indica is a marijuana plant found in India. It contains a class of unique chemicals collectively known as cannabinoids. Cannabinoids are found only in cannabis plants and have been found to be useful in the suppression of tumor cells. Apart from cannabinoids there are four hundred chemicals found in *Cannabis indica.* The leaf part of *Cannabis indica* is popularly known as bhang.

Different types of plants' extracts have been used as reducing agents for the synthesis of gold nanoparticles. Such types of plants include *Hibiscus rosa*, *Ocimum sanctum,* neem etc. Apart from these fruits extracts such as lemon, orange have also been used for the synthesis of gold nanoparticles. The leaf extract of bhang is used for the synthesis of gold nanoparticles.

Cannabis indica have also been found to be anti-cancerous [5]. The gold nanoparticles prepared using bhang can show better efficiency in the inhibition of proliferation of cancer cells.

1.6 Breast Cancer

In world breast cancer represents 9% of the global cancer burden and is the third most common tumor. In India one in 22 women during her lifetime is likely to suffer from breast cancer [6]. Current

therapy for breast cancer includes hormonal therapy and aromatase inhibitors. Due to growing resistance to these therapies search for new therapeutics for breast cancer has become essential. In our work we have used MDAMB-231 breast cancer cells. MDAMB-231 cancer was obtained in 1973 from a patient in M.D. Anderson Cancer institute and so it is named. This cancer cells are triple negative cancer cells.

In our present investigation we prepared gold nanoparticles through citrate reduction method [7] and by the reduction of bhang extract. The conjugates of gold nanoparticles and α-lac were prepared and they were test on MDAMB-231 cancels through MTT assay.

1.6 Objectives

- To synthesize and characterize monodispersed gold nanoparticles

- To prepare conjugates of gold nanoparticle-α lac protein

- To administer the conjugates in the MDAMB-231 cancer cells and check their cytotoxicity by MTT assay.

CHAPTER 2

Literature Review

2.1 Gold nanoparticles

Gold nanoparticles are conventionally synthesized by the reduction of $HAuCl_4$ by a reducing agent in presence of a stabilizer. Turkevich in 1951 first demonstrated this method while the synthesis of gold nanoparticles of controlled sizes by altering the concentration of reducing agent and stabilizing agent was demonstrated by Frens in 1973[8]. Since then the synthesis of gold nanoparticles by chemical reduction process has been carried out and modified through different approaches such as heating and stirring method, microemulsion method. Gold nanoparticles appear in ruby red color due to a phenomenon called Surface Plasmon Resonance which was first explained by Mie by solving Maxwell equations. The surface plasmon resonance is due to the resonance of incoming light with the surface electrons of nanoparticles which reflects light at the visible spectrum of wavelength of around 520nm. The surface plasmin resonance is not effective in nanoparticles of size less than 2nm due to the quantum size effect [8]. Gold nanoparticles of different shapes such as spherical, oblong, rod-like etc. can be synthesized by altering the method of preparation and parameters. Concentrations of reagents, temperature, pH, pressure, time of reaction are some of the important parameters which need to be controlled for the synthesis of gold nanoparticles.

2.2 Microemulsion

Microemulsions are thermodynamically stable homogenous mixture of oil and water containing structural units of sizes in the range of 3-30nm. The structural units are generally man-sized and dispersed within the medium of the emulsion. Microemulsions are currently characterized through various types of instruments which render a better understanding of the nature and sizes of the

structural units. The different techniques which are used to characterize microemulsion are Dynamic Light Scattering (DLS), Small Angle X-ray Scattering (SAXS), Nuclear Magnetic Resonance (NMR), Small Angle Neutron Scattering (SANS), Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM) [9]. Reverse micelles that are water droplets in dispersed oil phase microemulsions have been used to prepare gold nanoparticles of different sizes and shapes. Such micelles are stabilized by the addition of surfactant and co-surfactant. The micelles are globular in shape at low water content but its shape becomes rod-like at the addition of water. The size of the droplets is dependent upon the ration of water concentration to surfactant concentration. Reverse micelles can be used to synthesize nanoparticles by the method of chemical reduction or co-precipitation [10].

Gold nanoparticles of different shape, size and chemical properties can be synthesized by the use of microemulsion method. Apart from the size and radius of curvature of the droplets there are many other parameters such as chemical condition of environment, temperature of system can alter the size and shape of the nanoparticles formed.

2.3 α-lactalbumin protein

α-lactalbumin protein is a whey protein generally found in milk. It is divided into two domains viz. α-domain and β-sheet [11]. It is one of the most widely studied proteins. Its complex with oleic acid is known as HAMLET in case of human protein and BAMLET in case of bovine protein. BAMLET formation can take place by heat shock process. In this process the protein is mixed with oleic acid and incubated for ten minutes at 60°C. α-lac contains an amino acid which gives fluorescence upon

absorbance of UV light at 280nm. The name of the amino acid is tryptophan. Tryptophan is present in the hydrophobic core of the protein.

2.4 Synthesis of nanoparticles

Gold nanoparticles are widely synthesized by chemical reduction method. The reduction of gold from +3 oxidation state in auric chloride to 0 oxidation state takes place. A reducing agent is used for the reduction of gold. Normally tri-sodium citrate and sodium borohydride is used for the synthesis of gold nanoparticles. Apart from this gold nanoparticles have also been synthesized by microwave radiation. Red color spherical nanoparticles have been reported to form from the reduction process.

The red color is due to a phenomenon known as Surface Plasmon Resonance (SPR). In SPR when the incoming visible light strikes with the electron oscillating on the surface of the nanoparticle then at a certain wavelength a resonance takes place where the frequency of the light matches the frequency of the oscillation of the electron. The SPR effect takes place only in case of nanoparticles because for the electrons must be present on the surface of the nanoparticles. Moreover no SPR is observed in particles whose size is less than 2nm because then the quantum effect starts taking place [8].

2.5 Dynamic Light Scattering (DLS):

Dynamic Light Scattering works by scattering light from the particles or molecules and studying their Brownian state of motion and thereby measuring their size and geometrical structure [12]. The motion of the particles due to the collision of the solvent molecules around the particles is termed as Brownian motion. The velocity of Brownian motion is defined by translation diffusion co-efficient. The translation diffusion co-efficient determine the size of the particles by Stokes-Einstein equation [13].

$$D = \frac{KT}{3\pi\eta d}$$

Where, d (H) = hydrodynamic diameter

K = Boltzmann's constant, D=Diffusion co-efficient, η = viscosity and T = absolute temperature

The parameters upon which the translation diffusion co-efficient are dependent are surface structure of the particle, core of the particle, concentration of ions in the solution.

2.6 Scanning Electron Microscopy (SEM):

Scanning Electron Microscope is used to image and analyze materials of sizes that are less then micrometer range. The electrons are accelerated by the potential difference between cathode and anode. The electrons emitted are secondary electrons, back scattered electrons and auger electrons which decide upon the energy spectrum that is available by the interaction of electrons and specimen [14].

During SEM the signal is developed when electrons are interacted with the atoms that are at the surface of the material. The signals are secondary electrons, back scattered electrons, characteristic X-ray etc. [14].

2.7 U-V spectrophotometer:

Ultraviolet-Visible spectrophotometer is designed to use light of ultraviolet and visible spectral region. This technique is used to determine the concentrations of unknown solutions and determination of transition metals whose electron transition energy that fall under UV or visible regions. Beer-Lambert law is used to determine the concentration of unknown solutions. It states that the absorbance of light of a material is directly proportional to the concentration of material in solution and the path length of solution through which the light passes.

Chapter 3

Materials and Methods

3.1 Materials

3.1.1 Materials for gold nanoparticles and microemulsion

n-Heptane, methanol, propanol were obtained from Merck. CTAB, tri-sodium citrate, sodium dodecyl sulphate were obtained from Himedia, Oleic acid was obtained from RFCL limited and auric chloride hydrate was obtained from Sigma Aldrich. Sodium Borohydride and 1- Butanol was purchased from Merck. All the glass-wares were obtained from borosil and Millipore water was used in all preparations.

3.1.2 Instruments for analysis

UV-spectrophotometer was obtained from Perkin Elmer Lambda 35. Dynamic Light Scattering instrument used was obtained from Malvern while the Weighing balance was obtained from Precisa. Scanning Electron Microscope and Energy Dispersive X-ray of Jeol was used while biosafety cabinet of ESCO AC24E1 was used. Olympus CKX inverted microscope and Biorad microplate reader was used.

3.1.3 Chemicals for cell culturing and analysis

MBAMD-231 cancer cell lines were obtained from NCTS Pune, MTT kit and DMEM media were obtained from Himedia. α-lac was obtained from Sigma Aldrich.

3.2 Methods

3.2.1 Preparation of microemulsion

Water in oil type microemulsion is prepared by adding given amounts of oil, surfactant, water and co-surfactant. The water amount should be as less as about 100 times than the amount of oil. The water to surfactant concentration ratio would determine the structural size of water droplets.

E-1, E-2, E-3, E-4 are the experiments conducted in an attempt to prepare microemulsions.

E-1: 6mL of n-Heptane was taken and to it 0.24mL of oleic acid was added as surfactant.

0.06mL of water was added to it and the mixture was shaken for some time.

E-2: 25mL of n-Heptane is mixed with 1.33mL of 2-propanol, 1gm SDS and 0.69346mL of water.

E-3: 5mL of n-Heptane was mixed with 0.25mL of water, 0.78125mL of ethanol and 0.25gm of CTAB.

E-4: 6.4mL of n-Heptane was mixed with 0.32mL of water, 0.32gm of CTAB and 1.0mL of 1-butanol

3.2.2 Synthesis of gold nanoparticles by microemulsion

Normally there can be two ways to prepare gold nanoparticles through microemulsion viz. (i) Preparing the microemulsion containing reducing agent and then adding auric chloride aqueous solution and (ii) preparing two microemulsion, one containing auric chloride and the other containing reducing agent and then mixing them together.

100mM stock solution of auric chloride hydrate was prepared by adding 12.7mL of sterilized distilled water to the purchased 500mg of auric chloride hydrate. The glass wares were washed with aqua regia which is the mixture of hydrochloric acid and nitric acid in the ratio of 3:1 respectively which dissolve all the trace metals present in the glass wares as impurity. 10mM auric chloride solution was prepared using 0.9mL of distilled water ad 0.1mL of 100mM auric chloride stock solution.

Microemulsion E-4 was made by using 0.30mL of 10mM $HAuCl_4$ aq. solution, 0.30gm of CTAB, 1.0mL of BuOH and 6.0mL of n-Heptane.

Microemulsion E-4 was again prepared by mixing 0.30mL of 100mM $NaBH_4$ aq. solution,

0.30gm of CTAB, 1.0mL of BuOH and 6.0mL of n-Heptane. 4mL of E-4 prepared from $HAuCl_4$ aq. solution was magnetically stirred and to it 2mL of E-4 prepared from $NaBH_4$ aq. solution was titrated drop wise.

The gold nanoparticles prepared were characterized through DLS (Dynamic Light Scattering) and U-V Spectrophotometer.

3.2.3 Purification of nanoparticles:

The nanoparticles produced must be separated from the microemulsion in-order to be useful for biological application. The separation and purification is a tedious task. The purification can be done by the process of centrifugation and filtration.

In continuous microemulsions the phases can be separated by lowering the temperature of the mixture which can be useful for separation of nanoparticles suspension [15].

3.2.4 Synthesis of gold nanoparticles by *Cannabis indica* leaves extract

Cannabis indica leaves (common name in India is Bhang) acquired from wild were finely crushed in 5mL of water. The leaves were then centrifuged at 5000rpm for 10 minutes. The supernatant formed was filtered from 0.22µm syringe filter and pellet was discarded.

A set up was prepared for the synthesis of gold nanoparticles in order to prevent the escaping of water from the solution as water vapor and allow other gases to pass through or otherwise impurities and less water content may affect the size and morphology of nanoparticles drastically. The condenser was fitted with a motor in order to circulate water at 0°C. The temperature of the solution at 100°C was maintained through hot water bath which was placed on a heater cum magnetic stirrer (Figure 3). Required amount of $HAuCl_4$ solution was heated with CTAB solution. After two minutes the extract (supernatant) was added and then three minutes after that the color of the solution turned to black to

dark red. CTAB (surfactant) was added as a stabilizer as during its absence nanoparticles precipitated and larger particles were formed.

3.2.5 Synthesis of gold nanoparticles by citrate reduction

Gold nanoparticles were synthesized from citrate reduction method by adding Poly ethylene glycol (PEG) as capping agent.

Figure 3 Condenser fitted with two neck flask placed on a heater cum stirrer

3.2.6 Preparation of gold nanoparticle- α-lac protein conjugates

The stock solution of α-lac and BSA was prepared. The stock solution was of 38mg/11.5mL. Different concentrations of gold nanoparticles and α-lac protein have been mixed in PBS buffer and incubated the mixture at 37°C for 30 minutes and at 60°C for 10 minutes (Table 1) [16]. The conjugates were prepared by mixing gold nanoparticles synthesized from *Cannabis indica* leaves and citrate reduction method. BAMLET was prepared by heat shock method. The potential of separated conjugates as anti-cancerous therapeutics will be analyzed.

Conjugates Prepared
❖ **Gold nanoparticle (citrate reduction method)-α lactalbumin protein**
❖ **Gold nanoparticle (made from bhang)-α lactalbumin protein**
❖ **BAMLET**
❖ **Bhang-α lactalbumin protein**
❖ **Gold nanoparticle-Bovine Serum Albumin protein**
❖ **Bhang-Bovine Serum Albumin protein**

Table 1: List of the conjugates prepared

3.2.7 Preparation of cell growth medium

DMEM (Dulbecco's Minimum Essential Medium) was used for the culture of MDAMB-231 cancer cells supplemented with 10% Foetal Bovine Serum, 2mM glutamine and 0.1mg/mL streptomycin. All the materials were taken and the culture media was prepared and the MDAMB-231 cells were cultured.

3.2.8 Monitoring the effect of conjugates on MDAMB-231 cancer cells

100µL of conjugates have been administered to the breast cancer cell lines (MDA-MB-231) to monitor the effect of conjugates on the cancer cell lines 8 hrs after addition of conjugates. 10µL of MTT ([3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) was administered 2 hrs after the addition of conjugates and left for 6 hours. After six hours the media was removed and DMSO was added to dissolve the formazan formed. Formazan gets dissolved and forms a violet color. The absorbance was read at 595 nm. The absorbance values were recorded and the graph was plotted for estimation of viability of cells.

Chapter 4

Results and Discussion

4.0 Results and Discussions

4.1 Gold nanoparticles prepared from microemulsion method

The composition of the mixture E-1, E-2, E-3 prepared were taken on the hit and trial basis while E-4 was taken from a paper and the composition of auric chloride and sodium boro-hydride microemulsion solutions were taken on a hit and trial basis similar to E-4.

The microemulsion E-1 prepared was clear at the beginning but after 24 hours it was observed that the bigger water droplets were formed and settled down in the beaker which was due to flocculation which signified that the microemulsion was unstable. The observed phenomenon can be attributed to the fact that no co-surfactant was added to the mixture for stability and prevention of coalescence of water droplets.

The mixture E-2 prepared did not form any microemulsion because it was very thick and white in color which is the indication of formation of normal emulsion. Same result was found in E-3.

E-4 was clear which the indication of the formation of microemulsion was. The DLS of E-4 was done (Figure 4)

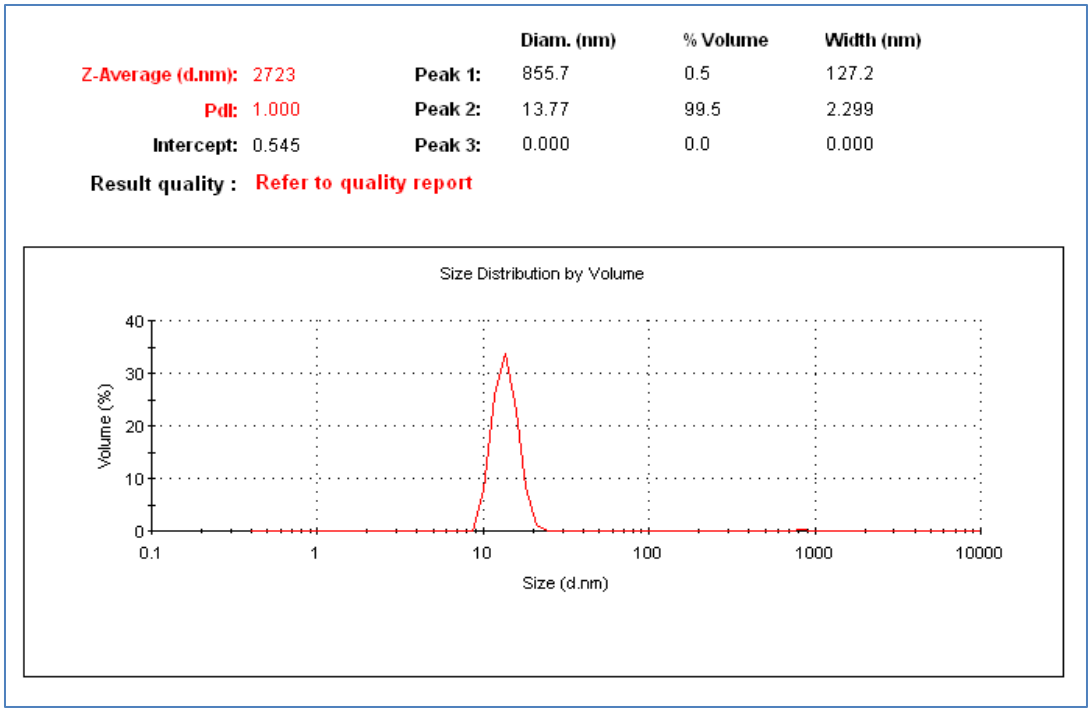


Figure 4 : DLS result of gold nanoparticles prepared from microemulsion process

The difference in the graph of DLS and the mentioned average size is due to the fact that DLS measures hydrodynamic diameter.

The gold nanoparticles prepared were red in color and its absorbance maxima were determined by U-V spectrophotometer (Figure 5). The absorbance maximum was found to be at 527.02nm which indicates that the gold nanoparticles were present based on the surface plasmon resonance and since the absorbance maxima was about 0.4 so the nanoparticles may be below 30nm in size. No presence of secondary peak indicated that the nanoparticles were spherical in shape.

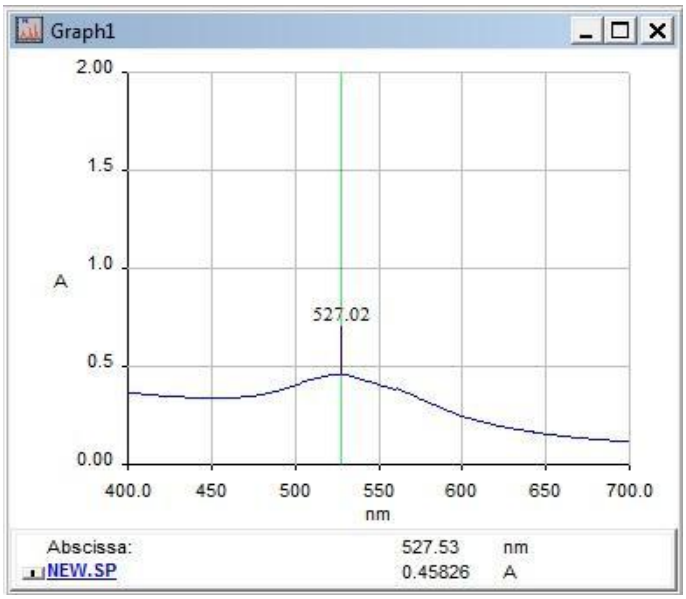


Figure 5 Absorbance maxima of gold nanoparticles measured by U-V spectrophotometer

The gold nanoparticles prepared from this process could not be separated from oil after many repeated attempts of centrifugation. So, a different method that is applying heat and mechanical energy will be employed for the synthesis of gold nanoparticles.

4.2 Synthesis of gold nanoparticles from heating-stirring method

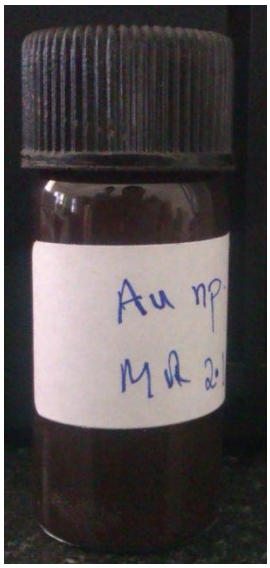


Figure 6 Gold nanoparticles prepared from bhang reduction method

Figure 6 shows the red color gold nanoparticles prepared from bhang reduction method. The UV-Vis analysis of the gold nanoparticles synthesized from bhang was done and its maximum absorbance was found to be at 586 nm (Figure 7). The blue shift was observed 30 days after the experiment and the maxima was found to be at 575 nm (Figure 7). And after 45 days the absorbance was found to be 568nm. This blue shift may be due to precipitation of larger particles or due to transfer of electrons from compounds in bhang (nucleophilic reagents) to the nanoparticles which brings a drastic change in Fermi level. The increase of the effective concentration of free electrons in the nanoparticles may be the reason of the of the bleu shift. The optical properties of colloidal metals can be influenced by change in the electron density of the nanoparticles[17].

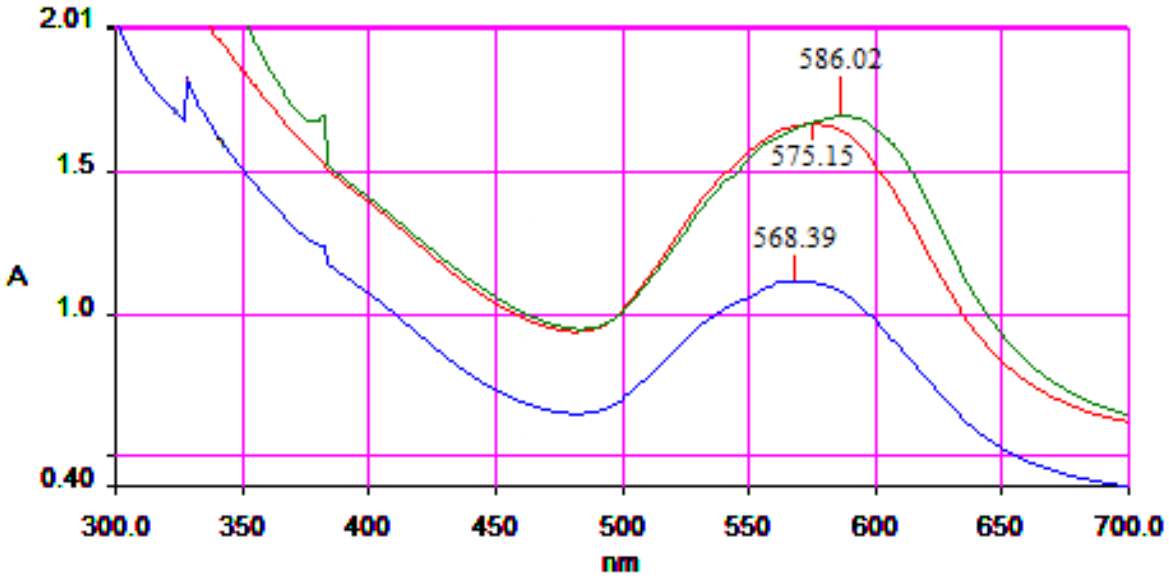


Figure 7 UV-Vis analysis of gold nanoparticles from bhang

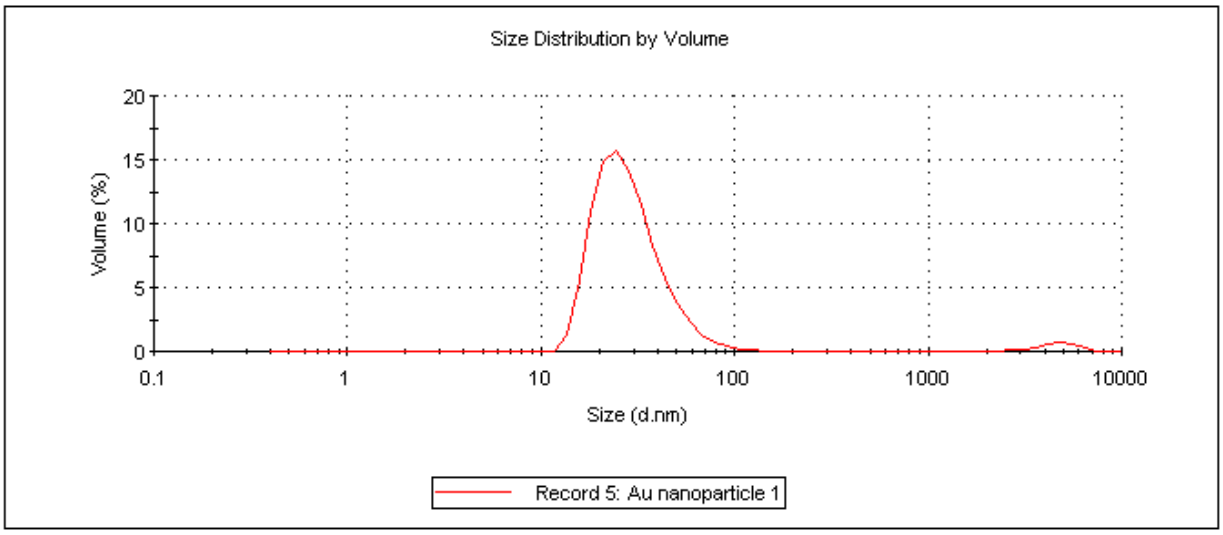


Figure 8 DLS analysis of gold nanoparticles synthesized from Citrate reduction method

Figure 8 represents the DLS analysis of the gold nanoparticles synthesized from the well-known Frens[7] method but here PEG was also added to increase its monodispersity and stability.

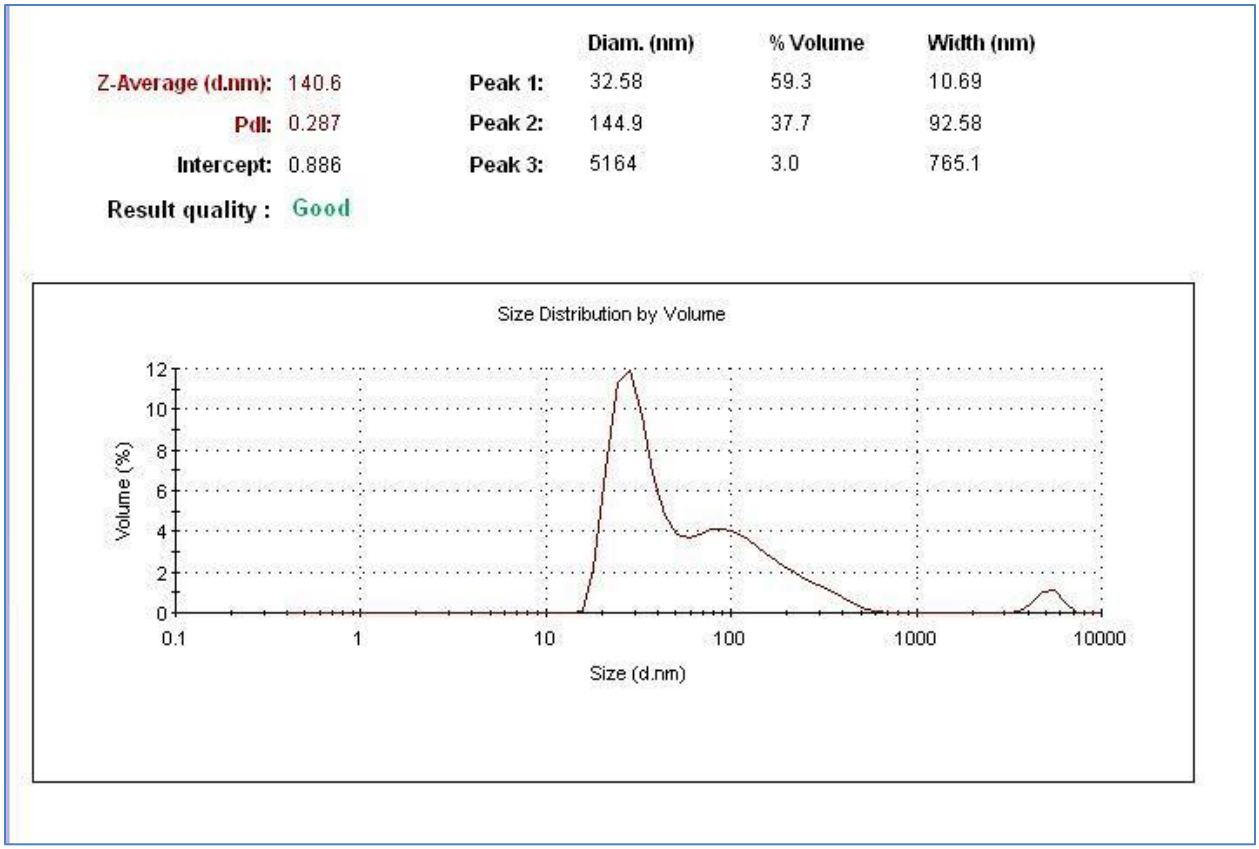


Figure 9 DLS analysis of gold nanoparticles synthesized from bhang reduction method

The DLS analysis of the gold nanoparticles synthesized by bhang reduction method was done (Figure 9). The average diameter of the gold nanoparticles was found to be 140.6nm. However, the highest concentration of the gold nanoparticles was found to be around 32 nm in a volume of 59%. The high average diameter is due to presence of 5000nm particles. The polydispersity index was found to be 0.287. Polydispersity index represents the ratio of particles of different size to total number of particles. More the polydispersity index less the monodispersed are the particles. 0.287 represents the particles are nearly monodispersed.

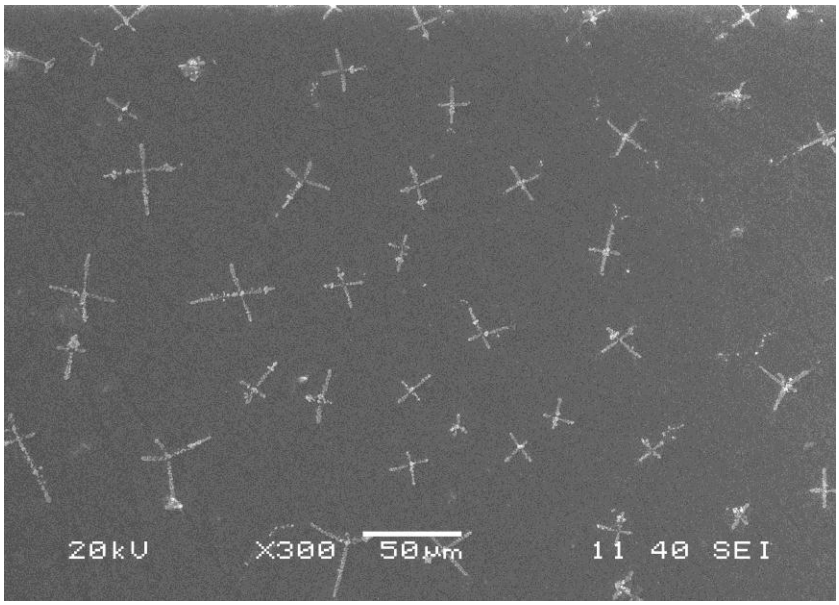


Figure 10 Self-assembly of gold nanoparticles in cross shaped from bhang reduction method

Self-assembled gold nanoparticles of cross shape (Figure 10) were formed from bhang reduction method. These types of self-assembly is formed when nanoparticles absorb any organic molecules. The molecules reduce the interfacial energy forming self-assembly. Self-assembled nanoparticles find huge applications in the field of nano-electronics, lithography and thin films [18]. The mechanism of formation of self-assembly is however not fully understood.

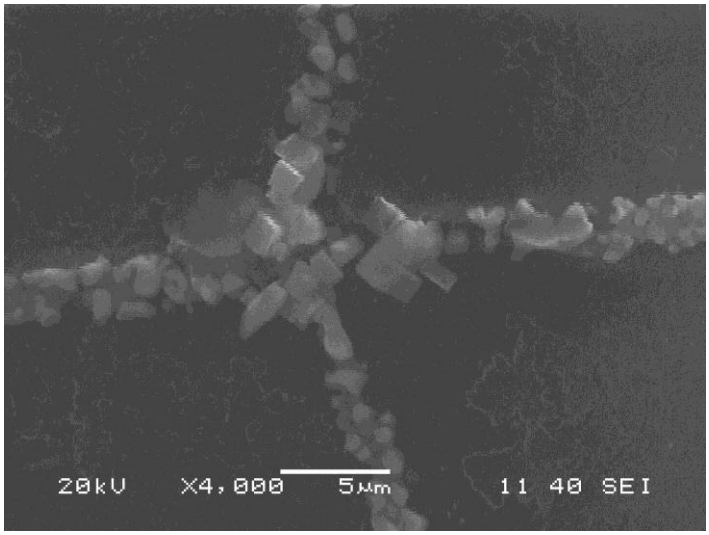


Figure 11 : Zoomed SEM graph of self-assembled gold nanoparticles

The zoomed graph of self-assembled gold nanoparticles shows that the shapes of the nanoparticles are not uniform. Rod shaped, spherical shaped and also cubic shaped nanoparticles were formed.

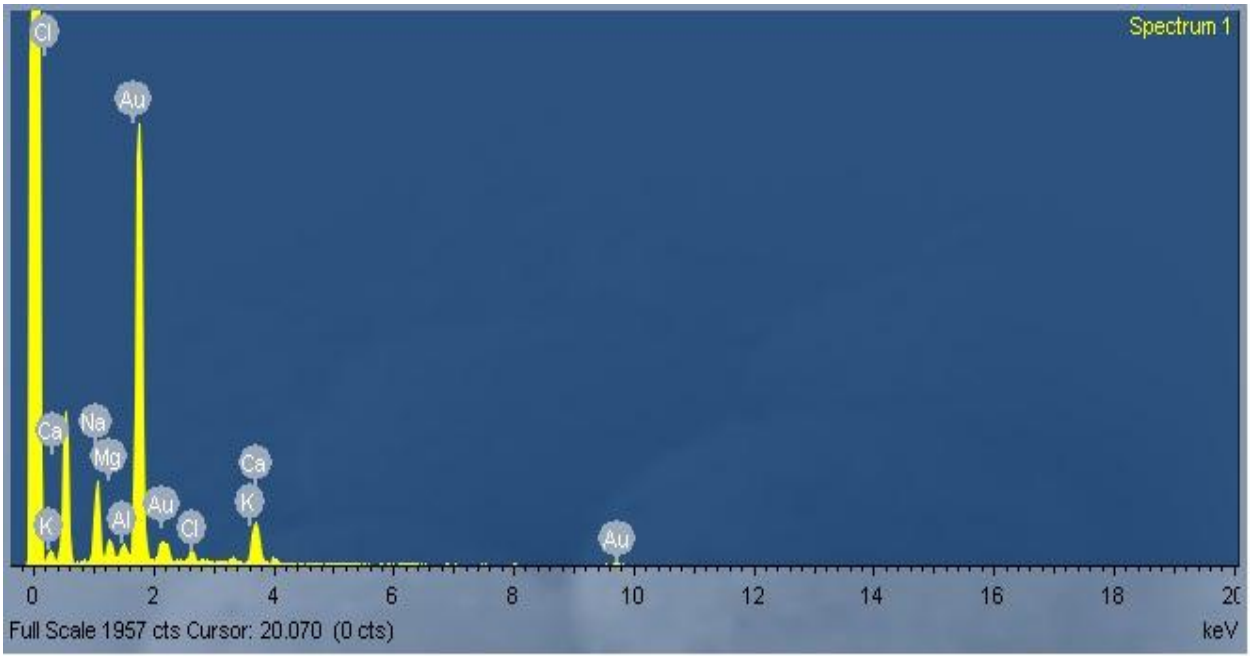


Figure 12 Energy Dispersive X-ray analysis of the gold nanoparticles

Energy Dispersive X-ray analysis (Figure 12) confirmed the presence of gold in the sample. Apart from gold many other metals were also detected like Magnesium, Calcium, Sodium and Potassium. All these elements might be present in the bhang extract. However, Aluminum was also detected which was due to the sample holder which is made from Aluminum.

4.3 Conjugates of gold nanoparticles and α-lactalbumin protein

Conjugates were prepared by mixing the appropriate amounts of solution so that in the final solution the concentration of α-lac remains 1mg/mL. Apart from the conjugates samples of pure gold nanoparticles prepared from citrate reduction method, bhang, Bovine Serum Albumin and only α-lac were also taken. Fluorescence spectra of all the samples were taken to see the change in fluorescence of tryptophan to see if any conjugate formation has taken place or not.

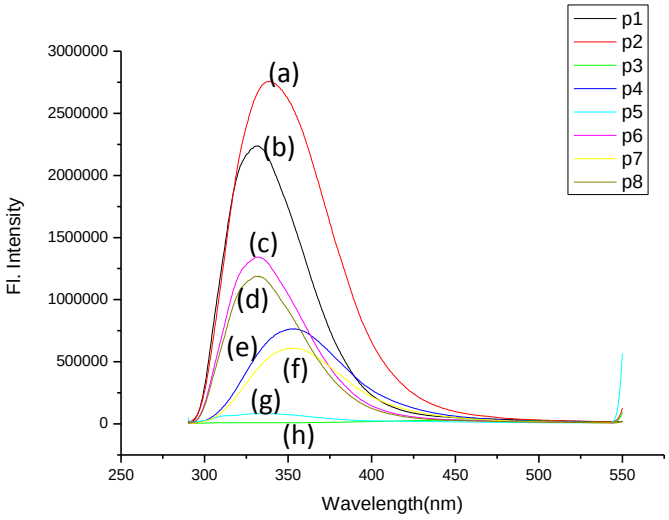


Figure 13 Fluorescence spectroscopy

(a) BAMLET (b) Native protein (c) Gold nanoparticles (citrate reduction method)-protein at 60° C (d) Gold nanoparticle (citrate reduction method)-protein at 37° C (e) Gold nanoparticle (bhang)-protein at 60°C (f) Gold nanoparticle (bhang)-protein 37° C (g) Gold nanoparticle (citrate reduction method) (h) Gold nanoparticle (bhang)

In all the cases by fluorescence spectroscopy analysis we have found that protein-gold nanoparticle interaction causes substantial structural changes of protein. In all cases, tryptophan fluorescence intensity of α-lac protein has been reduced and this is the indication of change of the environment surrounding tryptophan residues which resulted due to conformational change of the protein. In the case of gold nanoparticle synthesized in chemical synthesis method, their interaction with α-lac bring conformational changes of protein without substantial changes of polarity of tryptophan residues, however, *Cannabis indica* based produced nanoparticles bring such changes. This may, perhaps due to their specific shape (cross shaped self-assembly) and size or molecules of *Cannabis indica*. The whole set of experiment proves a stable conjugation formation between nanoparticles and protein.

4.4 Effect of conjugates on MDAMB-231 cancer cells

The conjugates were administered in the cultured MDAMB-231 cancer cells along with MTT and left for 8 hours. The effect of the conjugates was measured by taking the absorbance at 595nm through a microplate reader.

Serial no	Samples	% Inhibition
1	Blank	0.00
2	GNP (CRM)*	24.13
3	GNP*	61.00
4	GNP (CRM)-α lac	14.61
5	GNP- α lac	43.77
6	α-lac	38.52
7	BAMLET	46.12
8	GNP (CRM)-α lac 37°C	19.69
9	GNP- α lac 37°C	43.55

Table 2: % Inhibition of proliferation of cancer cells

From the data (Table 2) it is clear that preparation of conjugates at different temperatures won't affect the inhibition of proliferation of cancer cells. The inhibition caused by only protein is not enough as caused by the complex of gold nanoparticle and protein. The conjugates formed from

gold nanoparticle prepared by citrate reduction method did not caused as much inhibition as caused by complex prepared by gold nanoparticles from bhang reduction method. This proves that gold nanoparticles prepared by bhang have better efficiency to cause the inhibition of proliferation of MDAMB-231 breast cancer cells. However, it was observed that gold nanoparticles prepared by bhang reduction method along caused the highest inhibition. The reason of this was not understood so a second set of experiment was performed. This time bhang extract was also administered on the cancer cells as control of the complex. Moreover, a separate protein Bovine Serum Albumin was also used to prepare complex with gold nanoparticles to see if the conjugate of any protein with the nanoparticle can have same effect as α-lactalbumin protein.

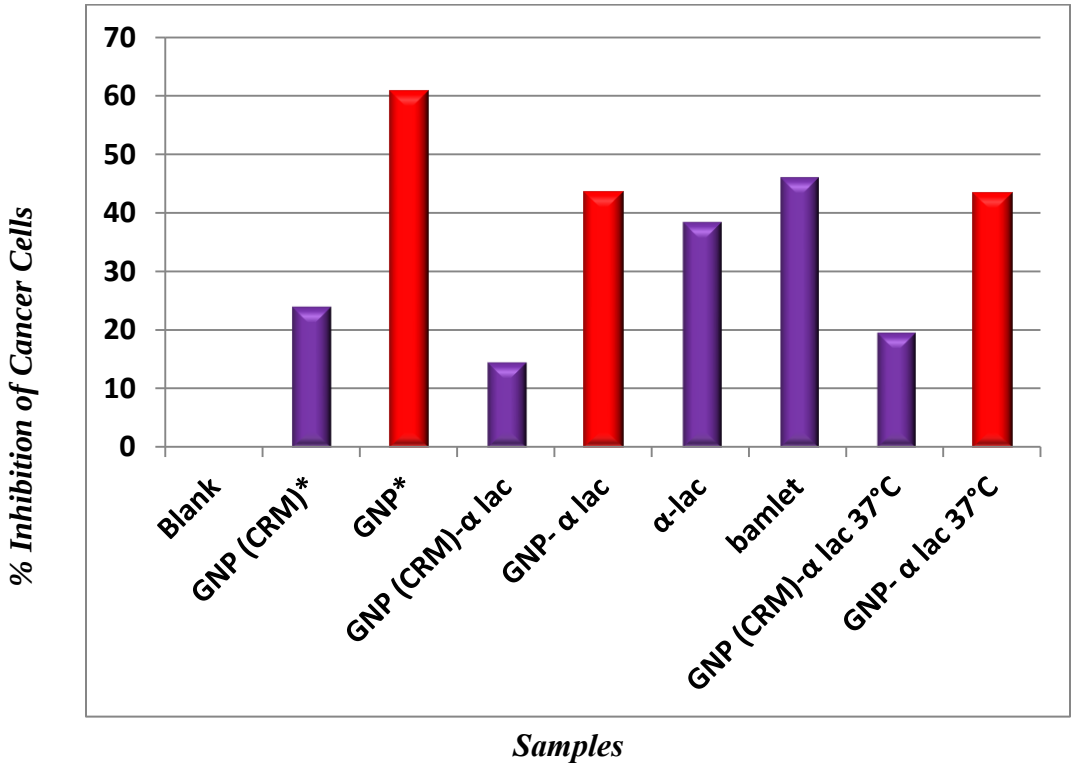


Figure 14 Graph of inhibition of cancer cells vs. samples

***GNP → Gold nanoparticles prepared from bhang reduction method**

***GNP (CRM) → Gold nanoparticles prepared from citrate reduction method**

Serial no	Samples	% Inhibition
1	Blank	0.00
2	BSA*	13.26
3	α lac	40.34
4	Bhang	36.17
5	GNP α lac	53.22
6	Bhang-BSA	32.77
7	GNP-BSA	31.25
8	Bhang-α lac	20.64
9	GNP	34.47

Table 3: % Inhibition of cancer cells by the conjugates and their controls

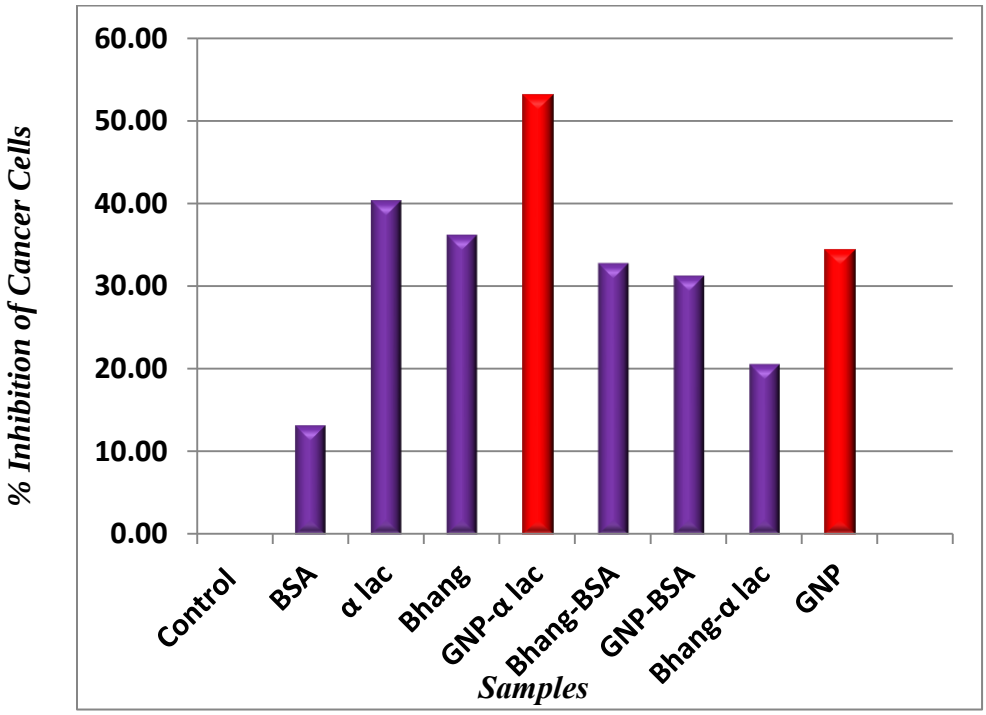


Figure 15 Graph of inhibition of cancer cells vs. samples

From the second set of experiments (Figure 15) its s clear that BSA does not play a significant role in the inhibition of the proliferation of the cancer cells. The conjugate of gold nanoparticle and α-lactalbumin protein again showed good inhibition of the proliferation of the cancer cells. However, this time the inhibition caused by gold nanoparticles alone was only 34.47% as compared to the previous value to 61%. This change in the inhibition of the cells can be explained by nanophotothermolysis [19]. In nanophotothermolysis when gold nanoparticles absorb visible light they emit heat energy. The temperature produced at that time for 30nm nanoparticles for a light of wavelength around 520nm is 2500K for 1-10nanoseconds. At this high temperature the cells die. The first experiment was performed during day light with the light of biosafety cabinet on while the second experiment was performed during evening when no electricity was there. So, during first experiment the gold nanoparticles were exposed the visible light which was not during the second experiment.

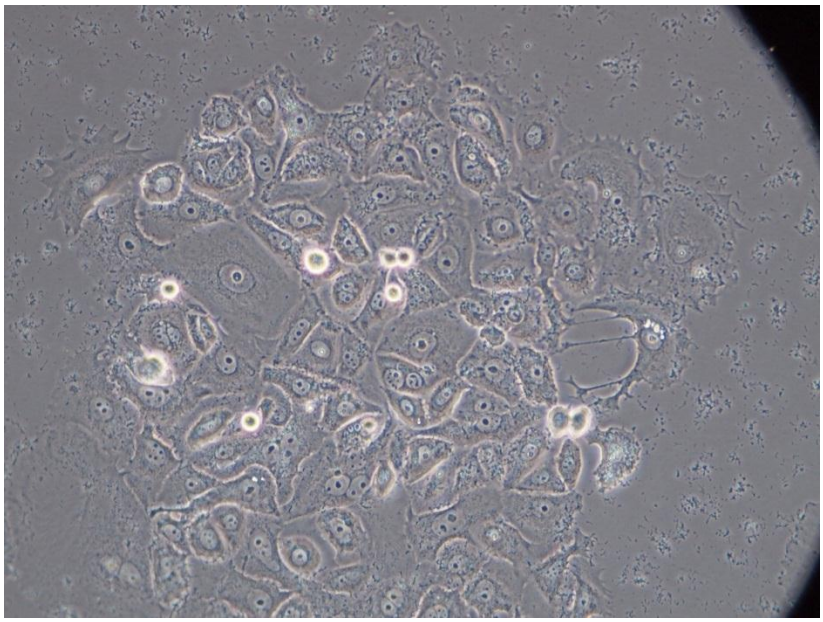


Figure 16 MDAMB-231 cancer cells before trypsinisation

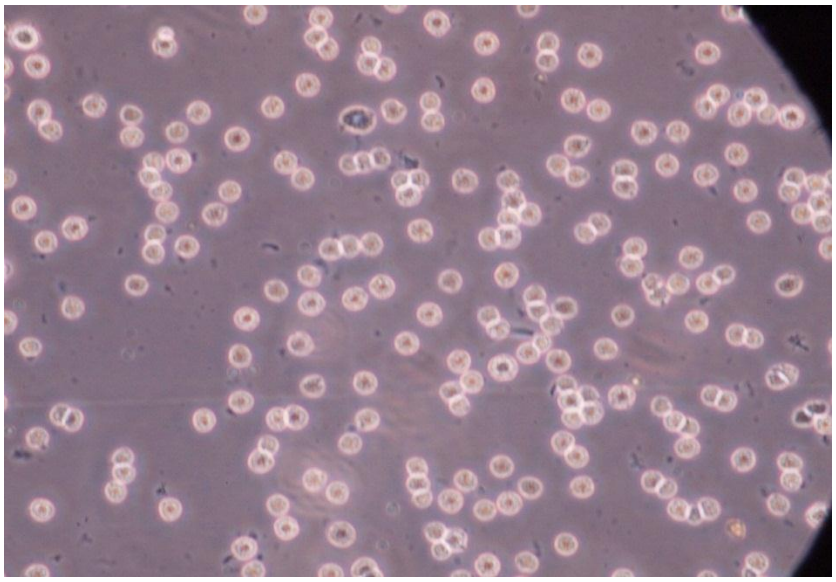


Figure 17 MDAMB-231 cancer cells after trypsinisation

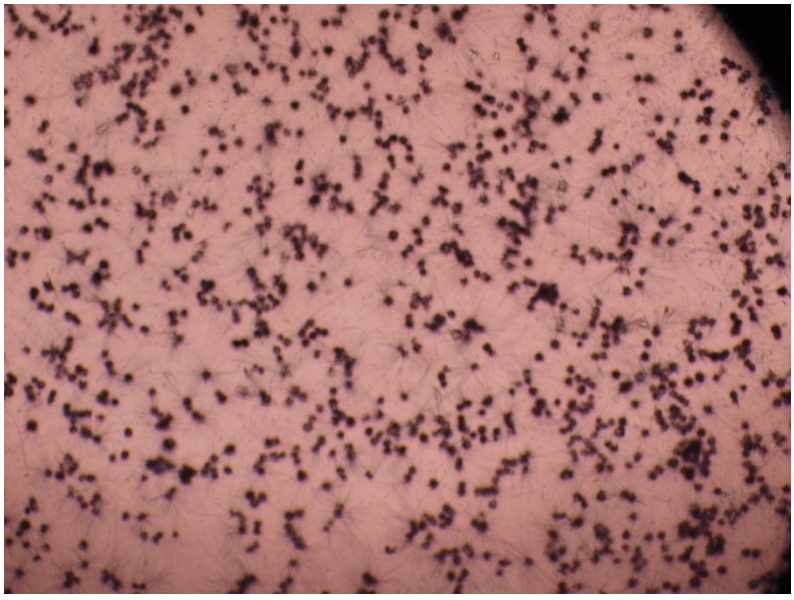


Figure 18 Blank after MTT assay

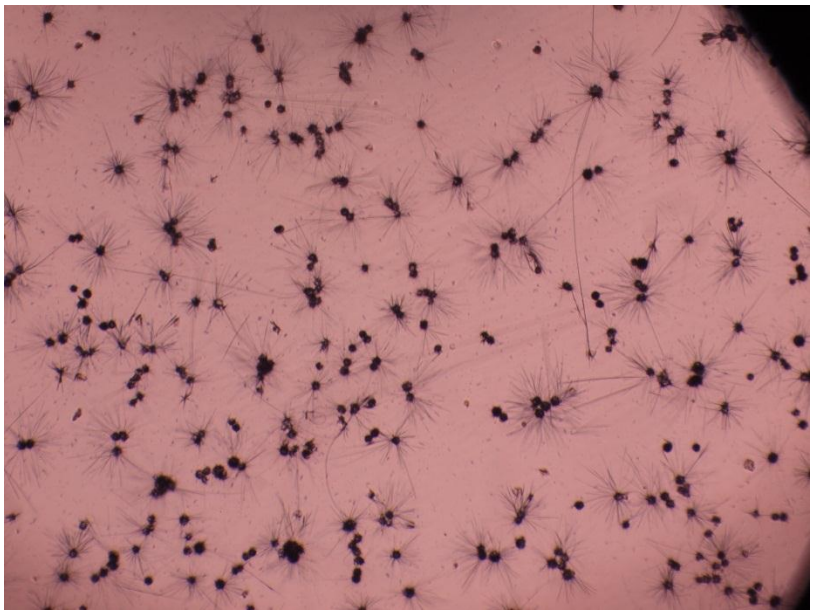


Figure 19 Cells administered with Gold nanoparticle-α lactalbumin protein complex after MTT assay

Chapter 5

Conclusion and Future work

5.1 Conclusion

Self-assembled gold nanoparticles can be prepared by the method of bhang (*Cannabis indica leaves*) reduction method. Bhang carries potential chemicals for the reduction of gold from hydrochloric acid. The use of CTAB produced sable gold nanoparticles where blue shift was observed over a range of 45 days.

The conjugates prepared from nanoparticles made from bhang reduction method and α-lactalbumin protein have potential for the inhibition of proliferation of MDAMB-231 breast cancer cells. Nanophotothermolysis was observed during the case of gold nanoparticles made from bhang reduction method when the nanoparticles were exposed to visible light. The mechanism of the inhibition of cancer cells are not known at present.

5.2 Future Work

The amount of *Cannabis indica* leaves' extract required for a particular size and shape of nanoparticles will be standardized. The time and dosage of the conjugates to be administered on MDAMB-231 breast cancer cells for controlled inhibition has to be standardized and the mechanism of the inhibition of the proliferation of breast cancer cells has to be determined.

Chapter 6

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